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Effect of 1,25-dihydroxycholecalciferol on impaired calcium transport by the sarcoplasmic reticulum in experimental uremia

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Effect of 1,25-dihydroxycholecalciferol on impaired calcium transport by the sarcoplasmic reticulum in experimental uremia. In the fragmented sarcoplasmic reticulum from skeletal muscle of rabbits with experimental uremia, defective calcium ion transport is found. An impairment of all parameters is observed (initial rate of uptake, storing capacity with and without oxalate, and concentrating ability). *In vivo* administration of 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$-vitamin D_3)(2 × 27 ng × kg of body wt^{-1} × day^{-1} and 6 × 27 ng × kg^{-1} × day^{-1}, respectively) improved the kinetic parameters. The low dose improved storing capacity, and the higher dose, in addition to the storing capacity, also corrected concentrating ability and the initial rate of uptake. It is concluded that active calcium transport in the sarcoplasmic reticulum is impaired by uremia and that this defect is responsive to the administration of 1,25-$(OH)_2$-vitamin D_3.

Effet du 1,25-dihydroxycholecalciferol sur l'altération du transport du calcium par le réticulum sarcoplasmique dans l'urémie expérimentale. Un défaut du transport du calcium est observé dans le réticulum sarcoplasmique du muscle squelettique de lapins atteints d'urémie chronique expérimentale. Une altération de tous les paramètres est observée (débit initial de captation, capacité de mise en réserve avec et sans oxalate, capacité de concentration). L'administration *in vivo* de 1,25-$(OH)_2$-vitamine D_3 (2 × 27 ng × kg^{-1} × $jour^{-1}$ ou 6 × 27 ng × kg^{-1} × $jour^{-1}$) améliore les paramètres cinétiques. La dose faible améliore la capacité de mise en réserve et la dose la plus élevée corrige, outre la capacité de mise en réserve, la capacité de concentration et le débit initial de captation. La conclusion est que le transport actif de calcium dans le réticulum sarcoplasmique est altéré par l'urémie et que ce déficit est corrigé par l'administration de 1,25-$(OH)_2$-vitamine D_3.

Uremic patients often suffer from a peculiar form of metabolic myopathy which is characterized by proximal muscle weakness [1]. Based on clinical observations, Stanbury hypothesized that a defect of calcium ion transport in sarcoplasmic reticulum (SR) may be present. Histochemical [2] and electrophysiologic studies (Senges, Ehe, Ritz: Uremic myopathy: Changes in contractile and membrane properties, in preparation) have also shown abnormalities of skeletal muscle in clinical and experimental uremia. It is well established that the regulation of muscular contraction and relaxation is calcium-dependent. It is believed that the sarcoplasmic reticulum brings about muscular contraction by liberating calcium ions to the sarcoplasm in response to the arrival at the muscle of a nerve impulse, and that it then causes muscular relaxation by sequestering the released calcium ions again by an active transport process. A defect of calcium ion transport by sarcoplasmic reticulum has been demonstrated in experimental uremia [3, 4]. The following paper provides evidence that 1,25–dihydroxycholecalciferol (1,25-$(OH)_2$–vitamin D), the active metabolite of vitamin D, can reverse the impairment of active calcium transport by the sarcoplasmic reticulum in experimental uremia.

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Methods

Inbred New Zealand rabbits (Graflich Degenfeld Schonberg'sches Rentamt; Versuchstierfarm Eybach, Geislingen, Germany) of either sex (body wt, 2 to 3 kg) were used for the experiments. Uremia was induced in the animals by removal of the right kidney under sodium pentobarbital anesthesia (Nembutal®, 30 mg/kg of body wt) using a transabdominal approach. At least two-thirds of the left kidney was destroyed by ligation of the branches of the left renal artery. The kidneys were decapsulated in order to preserve the adrenal glands. Penicillin (250,000 IU) was given i.v. immediately after the operation. Simultaneously, the control animals were sham-operated (decapsulation of both kidneys). The animals were kept in single cages and pair-fed after operation with commercially available artificial food (Mischfutter fur Kaninchen, J. Zahn Co., Hockenheim, Germany). The animals had free access to water and were killed five days after nephrectomy. During this time

serum urea rose from 29 ± 7 mg/100 ml (controls) to 186 ± 104 mg/100 ml, and serum creatinine rose from 1.10 ± 0.2 mg/100 ml (controls) to 4.1 ± 1.8 mg/100 ml (Technicon Autoanalyzer, model SMA 12). Serum calcium was 4.5 ± 0.8 mEq/liter (controls, 5.2 ± 0.5 mEq/liter), and serum potassium was 4.5 ± 0.9 mEq/liter (controls, 5.4 ± 0.6 mEq/liter).

1,25-$(OH)_2$-vitamin D_3, dissolved in alcohol and kept at 4°C in the dark, was administered *per os* according to the time schedule given in Table 1. Serum calcium concentrations decreased after nephrectomy (uremic, 4.5 to 0.8 mEq/liter; controls, 5.2 to 0.4 mEq/liter) and rose again in animals treated with 1,25- $(OH)_2$-vitamin D_3 (2 × 27 ng/kg of body wt × 24 hr). 25-(OH)-vitamin D concentrations, measured with the technique of Edelstein [5], were slightly higher in uremic animals than in control animals. Neither control rabbits (serum 25-(OH)-vitamin D, 38.2 ± 3.85 nmoles/liter) nor uremic rabbits (serum 25-(OH)-vitamin D, 55.14 ± 7.37 nmoles/liter) were vitamin D-depleted.

Two dose levels of 1,25-$(OH)_2$-vitamin D_3 were chosen: a low dose of 2 × 27 ng/kg of body weight × 24 hr for the period of 5 days, and high dose of (6 × 6 × 27 ng/kg of body weight × 24 hr for a period of five days.

Preparation of sarcoplasmic reticulum. The experiments designed to evaluate the effect of 1,25-$(OH)_2$-vitamin D_3 in experimental uremia were performed using one uremic animal, one uremic animal treated with 1,25-$(OH)_2$-vitamin D_3, and one sham-operated control animal. To study any possible effect of 1,25-$(OH)_2$-vitamin D_3 on the controls, additional paired experiments were performed comparing one sham-operated control with one sham-operated control receiving the high dose of 1,25-$(OH)_2$-vitamin D_3. The sarcoplasmic reticulum was isolated according to De Meis and Hasselbach [6] from the leg and back muscles, the control and uremic animals yielding the same amount of vesicles per gram of wet muscle. The entire preparation procedure was performed in the presence of 10^{-4}M pheynylmethyl sulfonylfluoride (PMSF), an inhibitor of proteinases, especially trypsin and chymotrypsin. This has been done because tissue proteinases are known to be more active in certain pathological conditions. The vesicles were kept at 4°C and were used within two days after preparation.

Presence of a higher degree of non-sarcoplasmic reticulum protein and mitochondrial impurities in the "uremic" preparation were excluded by *1)* polyacrylamide gel electrophoresis [4], *2)* electron micro-

Table 1. The effect of 1,25 $(OH)_2$-vitamin D_3 on SR calcium transport parameters in experimental uremia[a]

	Initial rate of uptake[b]	Concentrating ability		Storing capacity[e]	
	nM Ca · mg⁻¹ protein · min⁻¹	*nM calcium*[c]	*Joule's · mole⁻¹*[d]	5 mM oxalate	no oxalate
				nM Ca · mg⁻¹ of protein	
Uremia, $N = 6$	1870 ± 536[h]	20.5 ± 7.9[h]	12083 ± 829[h]	3253 ± 733[h]	254 ± 88
Uremia, low dose 1,25[f] $N = 6$	1885 ± 805	20.5 ± 6.5	12083 ± 626	3643 ± 581[i]	332 ± 62
Control, $N = 6$	2685 ± 402	14.1 ± 3.7	12888 ± 626	5158 ± 1020	354 ± 198
Uremia, $N = 6$	2217 ± 296[h]	12.9 ± 4.5[h]	13127 ± 835[h]	6044 ± 1548[h]	501 ± 129
High dose 1,25[g], $N = 6$	2730 ± 322[i]	9.0 ± 1.9[i]	13885 ± 489[i]	8243 ± 2494[i]	542 ± 110
Control, $N = 6$	2768 ± 514	11.1 ± 4.8	13500 ± 920	7320 ± 1607	662 ± 317
Control, $N = 6$	2768 ± 517	11.4 ± 5.8	13482 ± 991	5535 ± 1859	470 ± 88
Control, high dose 1,25[g], $N = 6$	2984 ± 670 NS	9.2 ± 1.3 NS	13813 ± 323 NS	6219 ± 2157 NS	368 ± 156 NS

[a] The Wilcoxon test was used to evaluate paired differences; all experiments were performed using three (rsp. two) animals in parallel as described under "Preparation of sarcoplasmic reticulum."

[b] Rate of calcium uptake expressed is within first 30 sec after start of the reaction; the reaction was linear from 0 to 2 min, as demonstrated previously (Fig. 3)[4].

[c] Concentrating ability is expressed by the free calcium concentration in the incubation medium after 20 min of calcium uptake. Incubation medium contained EGTA (0.2 mM) and the reaction was started by addition of vesicles; pH = 7.0. Free calcium concentration was calculated from the remaining radioactivity in the incubation medium and the Ca-EGTA complex constant after Schwarzenbach [29].

[d] Maximum transmembrane osmotic gradient built up by the vesicles based on the values from footnote c and on an apparent solubility product of calcium-oxalate of 2×10^{-8} [8], which in the presence of 5 mM oxalate results in a free calcium concentration inside of 4×10^{-6} M.

[e] Storing capacity expressed by the amount of calcium taken up within 20 min of reaction. The assay contained excess calcium (0.2 mM), 5 mM oxalate as the precipitating anion (rsp. no precipitating anion) and 0.01 mg of vesicular protein per ml; pH = 7.0.

[f] Low dose = 2 × 27 ng/kg × 24 hr.

[g] High dose = 6 × 27 ng/kg × 24 hr.

[h] Difference between uremia and control, $P < 0.05$.

[i] Difference between uremia and treated, $P < 0.05$.

scopy, and *3)* incubation with sodium azide, which is known to inhibit mitochondrial ATPase activity [7].

Calcium kinetics. The kinetic studies were performed by simultaneous incubation of control sample and experimental sample(s) at room temperature using the following standard conditions [8]: 5 mM adenosine triphosphate; 10 mM magnesium chloride; 20 mM histidine; 40 mM potassium chloride; 5 mM oxalate; 0.1 mM calcium (+^{45}Ca); 5 mM phosphoenolpyruvate (PEP); 50 μg of pyruvate kinase/ml; 0.05 mg of vesicular protein per ml; pH, 7.0; ionic strength (μ) = 0.1. Specific conditions of incubation are given in the figure legends. The reaction was stopped by filtration of aliquots through millipore filters (Schleicher und Schull, Selectron-Filter, Type BA85; pore size, 0.45μ), and the difference between the radioactivity of the filtrate and the initial activity of the incubation medium was used to calculate the kinetic data.

Statistics. The difference between the paired control animals and experimental animals was evaluated with the use of Wilcoxon's test for paired differences. The figures are given as $\times \pm$ SD.

Electron microscopy. For electron microscopy, samples of SR preparations from control, uremic, and 1,25-$(OH)_2$-vitamin D_3-treated uremic animals were negatively stained on collodion and carbon coated grids according to standard procedures [9]. They were then observed in the electron microscope within a few minutes after air drying. A 1% potassium phosphotungstate aqueous solution adjusted to a pH of 7.0 with potassium hydroxide (KOH) was used. An electron microscope (Siemens Elmiskop, Model 101) equipped with a specimen cooling device was used with a double condenser illumination, 300 μm Pt and 30 μm Pt condensers and 30 μm Pt objective aperture, accelerating voltage of 80 kV and an emission current of 20 μÅ. Pictures were taken at a magnification ranging from $\times$10,000 to $\times$40,000.

Results

It has been shown that the vesicle membrane not only is able to transform a chemical potential into an osmotic potential [10] but also can transform an osmotic potential back into a chemical potential [11]. The whole transport reaction can be described stoichiometrically by the following equation:

$$2\,Ca_o + ATP + C_o \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} 2\,Ca_i + ADP + C_i + P_i + H^+ \qquad (1a)$$

where C is the hypothetical transport unit carrier; indices "o" and "i" represent outside and inside the membrane, respectively; P_i represents orthophosphate. For each mole of adenosine triphosphate (ATP) split, two moles of calcium ions are transported [8]. The energy linked calcium flux rates through the membrane thus can be described by:

$$\dot{V}_{Ca\ flux} = Ca_o^2 \times ATP \times C_o \times k_{+1} - Ca_i^2 \times ADP \times P_i \times C_i \times k_{-1} \qquad (1b)$$

Including the electrochemical terms, which, however, are unlikely to contribute to the energetic balance [12], the relation between osmotic and chemical potential at equilibrium then can be described by:

$$e^{\frac{V \times F}{R \times t}} \times \frac{Ca_i^2}{Ca_o^2} = \frac{ATP \times C_o \times k_{+1}}{ADP \times P_i \times C_i \times k_{-1} \times H^+} \times k_{ATP} \qquad (2)$$

where R = gas constant, T = absolute temperature (K^0), V = difference of electrical potential, F = Faraday constant, and k_{ATP} = equilibrium constant of ATP. (See definitions of equation 1 for C_o, C_i, Ca_o, Ca_i, k_{+1}, k_{-1} and P_i.)

Two groups of kinetic parameters can be distinguished: first, parameters determined in the absence of calcium uptake equilibrium (initial kinetics); and second, parameters determined in the presence of calcium uptake equilibrium (steady state kinetics). From equations 1b and 2 it can be seen that the principal factors which can affect transport kinetics are either the number of carrier transport units, C_o (outside) and C_i (inside), and/or the rate constants, k_{+1} and k_{-1}. Comparison of equations 1b and 2 shows that both factors influence both the initial and steady state kinetics. Therefore, an alteration of one factor should result in a change of all kinetic parameters. In the presence of high osmotic gradients, the kinetics of the membrane are additionally affected by passive calcium ion fluxes. Thus, the rate constant, carrier population, and passive calcium ion permeability ultimately characterize the membrane transport properties.

In uremia it was found [4] that all transport parameters are impaired. The present communication describes the effect of 1,25-$(OH)_2$-vitamin D_3 on transport kinetics in uremic animals.

Initial rate of influx. In Figure 1, the calcium ion exchange of control and uremic membranes is compared after preequilibration in the absence of net calcium ion uptake, i.e., during steady state. It can be seen that calcium ion exchange of the uremic membranes is slower than that of the control membranes. Defining the influx rate constant (K_i) by the terms given above

$$K_i = C_o \times k_{+1} = V_{Ca}\ \text{influx at equilibrium}/Ca_o^2 \times ATP, \qquad (3)$$

it was calculated [4] that the influx rate constant of

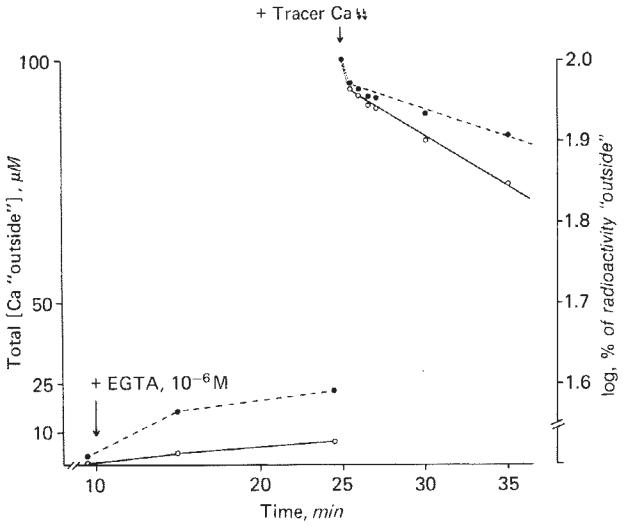

Fig. 1. *Calcium exchange at steady state of calcium uptake.* The graph gives one typical experiment. Assay conditions were chosen as described under Methods. The initial calcium concentration in the medium was 100 μM; ^{45}Ca was added to give an initial counting rate of 20000 counts/min/ml. After 10 min of preincubation, 0.1 mM EGTA was added to the assay (left side of the graph). After another 15 min of equilibration, when steady state conditions were established as confirmed by repeated measurements of ^{45}Ca, a small amount of ^{45}Ca of high specific activity was added in tracer amounts (right side of the graph). The influx rate constant was calculated [4] from the slope of the disappearance of activity (ordinate to the right), neglecting the first component (steep decline) which is thought to reflect membrane binding. The *solid line* represents vesicles from control rabbits; the *broken line* represents vesicles from uremic rabbits.

the uremic membranes is decreased. This decreased influx rate constant is consistent with the decreased initial influx rate of the uremic membranes (see Table 1).

The initial influx rate measured under nonsteady state conditions [8] was not consistently normalized by the low dose of 1,25-$(OH)_2$-vitamin D_3, but increased significantly after *in vivo* administration of the higher dose of 1,25-$(OH)_2$-vitamin D_3. In the dose given, 1,25-$(OH)_2$-vitamin D_3 failed to affect significantly the initial influx rate of the vesicles of nonuremic animals. Finally, the addition of 1,25-$(OH)_2$-vitamin D_3 to the incubation medium *in vitro* at a concentration of 0.027 ng/ml and 0.54 ng/ml was without any effect on the initial rate of influx.

Concentrating ability. As documented in Table 1, the rate of calcium ion uptake is diminished in uremia. In addition, a previous communication described an increased passive efflux [4]. These two changes explain why concentrating ability, i.e., the capacity to lower ambient calcium ion concentration, is diminished in vesicles of uremic animals. A measure of concentrating ability is given in Table 1 by the free calcium ion concentration "outside" after cessation of net calcium ion uptake and by the corresponding osmotic potential. Because of the use of an ATP regenerating system, the chemical potential in the control and uremic assay is identical. The osmotic potential, however, built up from this chemical potential, is approximately 10% less in uremic membranes compared to the controls. Concentrating ability was not significantly affected by the administration of the low dose of 1.25-$(OH)_2$-vitamin D_3. In contrast, the administration of the high dose of 1,25-$(OH)_2$-vitamin D_3 increased significantly the concentrating ability of the vesicles of uremic animals but failed to affect significantly the concentrating ability of the vesicles of nonuremic animals.

Storing capacity. Storing capacity is a measurement of the maximal amount of calcium taken up by the vesicles in the presence of excess calcium and ATP. This parameter is determined after cessation of net calcium ion uptake in the presence of a very low osmotic gradient. It therefore depends neither on the influx rate constant nor to a great extent on the permeability to calcium ions; it does depend, however, on vesicle geometry and/or vesicle leakiness, e.g., incomplete sealing. Storing capacity was markedly diminished in the uremic preparations and was increased by the *in vivo* administration of 1,25-$(OH)_2$-vitamin D_3, both in low and especially in high doses (Table 1).

Electron micrographic studies. Figure 2a shows a negatively stained preparation from the SR of a control rabbit. As reported previously [9], vesicular structures of various sizes and shapes can be observed. The vesicles are covered either with fine 40 Å particles or exhibit an electron dense rim. On the average the vesicles from the uremic nontreated rabbit (Fig. 2b) appear smaller than those from the control. The fine surface structure of the vesicle preparation from the uremic nontreated rabbit, however, does not appear to be altered. No significant difference between the SR preparations from control and 1,25-$(OH)_2$-vitamin D_3-treated uremic animals (Fig. 2c) could be found. Scattered open vesicles, mitochondrial fragments, and filamentous structures can be detected only occasionally in all three preparations.

Discussion

In uremia, it could consistently be shown that all parameters of active calcium ion transport in the sarcoplasmic reticulum (SR) of skeletal muscles are altered, the effect being responsive to 1,25-$(OH)_2$-vitamin D_3. These findings provide an interesting *in vitro* model to study the effects of uremia on an active cation transport system.

Exclusion of experimental artifacts. An increase of

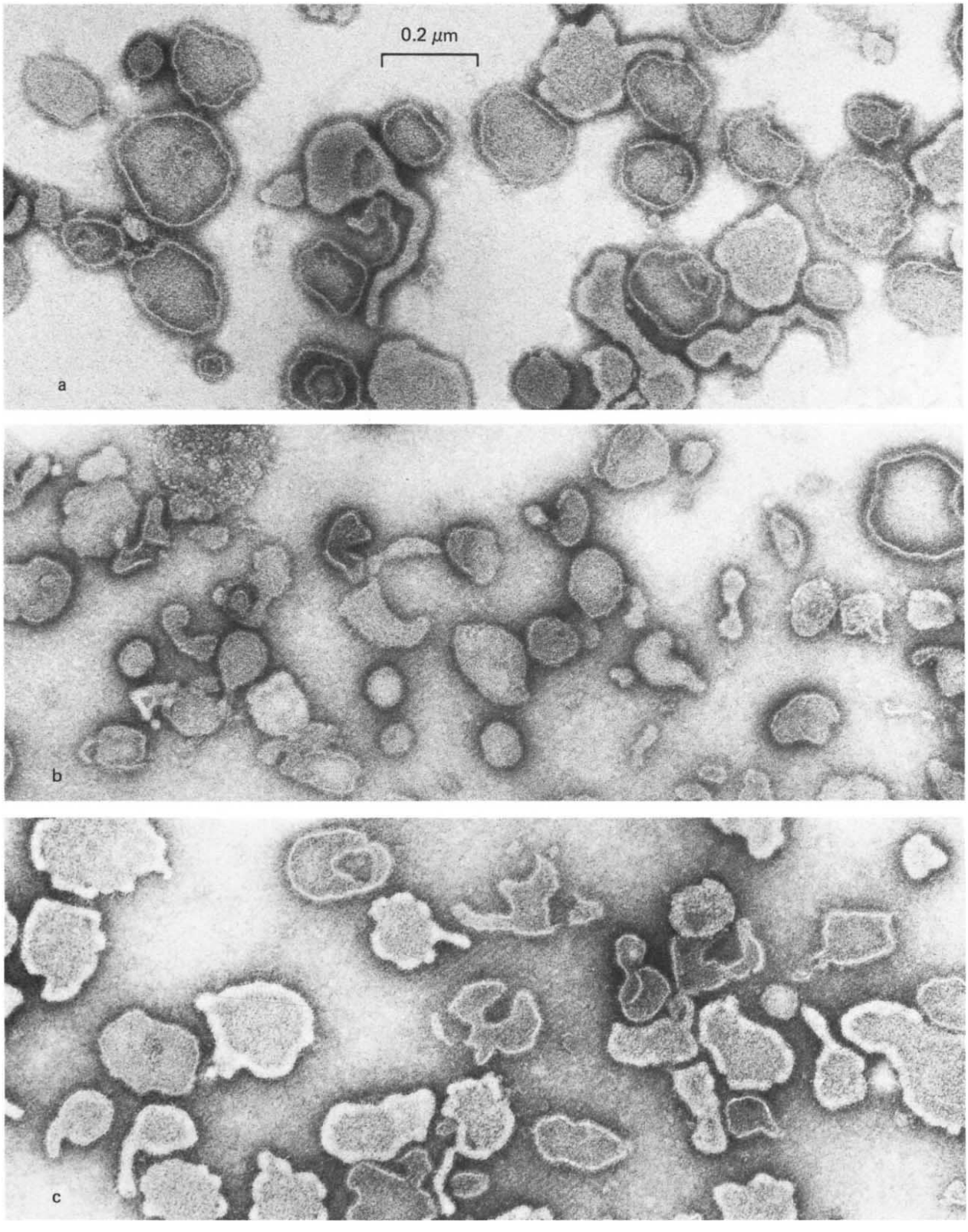

Fig. 2. *Electron micrographs of negatively stained SR vesicles from control,* **a**; *uremic,* **b**; *and 1,25-DHCC treated uremic,* **c**, *rabbits.* In **b**, vesicles appear on the average smaller than in **a**. Negative staining was done with 1% potassium phosphotungstate, pH, 7.0; ×80,000.

non-SR material, especially mitochondria, within the uremic preparation could be excluded by gel-electrophoresis, sodium azide, which showed no inhibition of the ATPase activity, and by electron microscopy. The impairment of the kinetic parameters is not likely to result from an eventual tryptic destruction of the uremic membranes since a trypsin inhibitor (10^{-4}M phenylmethyl sulfonylfluoride) was added to all the steps of the vesicle preparation. Also, less complete sealing of the uremic vesicles during the preparation procedure is unlikely, since differential centrifugation of uremic vesicles after completion of calcium ion uptake failed to show a higher proportion of vesicles with little or no calcium ion uptake ability compared to the controls. The presence of inhibitors in the assay system is unlikely, since a possible inhibitor should be lost during the preparation procedure with several washing steps.

Histological studies of Bundschu, Suchenwirth, and Durr [2] showed that the relative number of white, type II fibers of skeletal muscles is decreased in uremia, while a relative increase of red, type I fibers and of intermediary fibers occurs. A relative increase of vesicles from red muscle fibers within the uremic preparation should be reflected by an increased calcium-independent ATPase activity of the uremic preparation [13]. Such an increase, however, was not observed in the system under study [4]. It is known that the vesicles tend to form clusters at higher protein concentrations. This had the consequence of diminishing the relative calcium ion flux rates, calculated per mg of protein, by diminishing the transporting surface. Kinetic studies at different protein concentrations revealed the same relative decrease of calcium transport in vesicles of uremic animals. In addition, reduction of the transporting surface would fail to explain the change of transport ratio observed in previous experiments [4]. Recently, it has been suggested that phosphorylase kinase may have an activating effect on calcium ion transport [14]. The impaired calcium ion kinetics are most likely not the result of less phosphorylase kinase in the uremic preparation, since the ATPase activities of both preparations did not differ [4] and, furthermore, since significant changes of the gel-electrophoretic pattern of both preparations in regard to non-SR proteins were not observed. The defect observed, therefore, must be due to a direct change of vesicular membrane properties.

Transport kinetics. Net uptake of calcium ions can be regarded as a result of active inward transport and passive diffusional outward loss along osmotic gradients (pump-leak model). Three possible models for diminished net inward flux of calcium ions can therefore be envisaged as: *1)* diminished unidirectional influx, *2)* increased outward leak, *3)* both diminished influx *and* increased leak.

The passive efflux observed in the presence of high osmotic calcium ion gradients [4] points to an increased passive calcium ionic permeability of the uremic membranes. The initial rate of calcium ion uptake is measured at high calcium concentrations in the incubation in the absence of ethylene glycol bis (β-aminoethyl ether)-N, N tetraacetic acid (EGTA), thus establishing a minimal osmotic gradient at the beginning of the calcium ion uptake reaction. As shown previously [4], in the presence of a small osmotic gradient, the passive efflux from the uremic vesicles cannot possibly account for the diminution of net inward flux, since the initial rate of uptake of uremic vesicles was shown to be lower by an average of 556 nM calcium/mg of protein, while the passive efflux rate, measured under the optimal conditions of a *high* osmotic gradient, was increased in uremic vesicles only by an average of 5 nM calcium/mg of protein $\times$ min. Furthermore, energy dependent efflux is unlikely to be markedly different in the uptake experiment, since under controlled conditions of identical chemo-osmotic potential, energy dependent efflux was not found to be significantly different between uremic and control vesicles [4]. Therefore, decreased unidirectional influx can not be explained by increased active or passive calcium ion effluxes during uptake, but instead by anyone or all of the following: a decrease of the rate constant, a decrease of the number of transport sites, uncoupling between transport ATPase and calcium transport.

Uncoupling between ATP breakdown and calcium transport is suggested by the findings reported in a previous paper [4]. Since passive calcium ion fluxes through the membrane cannot pass through its lipoid moiety [15], the increased passive calcium ion efflux observed in the uremic membranes indicates an alteration of the protein moiety of the membrane due either to an altered primary structure and/or to altered lipid-protein interactions. An alternative explanation would be a change in vesicle size, suggested by our electron micrograph studies, which by virtue of increasing surface/volume ratio might provide for an increase of passive calcium ionic permeability. Storing capacity, a kinetic parameter largely independent of either influx rate constant or calcium ionic permeability, might also be affected by change of vesicle leakiness (e.g., incomplete sealing) or vesicle geometry. Relating to the influence of vesicular geometry, loaded vesicles of normal animals were centrifuged through a discontinuous sucrose gradient after completion of net calcium ion uptake (unpub-

lished experiments). The vesicles of the pellet fraction, which presumably had the highest calcium ion content were found to have the smallest diameter when evaluated by electron microscopy. The results of the kinetic experiments may be interpreted thermodynamically by stating that the rate and efficiency of calcium ion transport, i.e., the conversion of chemical potential to osmotic potential, is diminished. This is indicated by the calculations given in Table 1.

Electron microscopy. No tendency of individual SR vesicles to cluster could be observed in the uremic preparation. The vesicular membrane does not appear to be altered with the applied technique, and only a reduction in the vesicular size can be detected. As has been shown in other pathological conditions [16], this might possibly reflect a greater nonspecific fragility of SR from uremic animals, the result being the formation of smaller vesicles during the preparation procedure. No significant changes could be observed on the preparation of 1,25-$(OH)_2$-vitamin D_3-treated uremic animals, which, in agreement with the biochemical results, suggests that the uremic alteration may be prevented by treatment with 1,25-$(OH)_2$-vitamin D_3.

Relevance to in vivo muscle function. Impaired calcium ion uptake was observed not only in the presence of oxalate, a nonphysiological precipitator of calcium ions, but also in the presence of phosphate [3], approximately 2mM of which has been estimated to be present in skeletal muscle [17]. The results, however, do not necessarily imply that the defect observed *in vitro* can manifest itself in disturbed muscle relaxation *in vivo*. Experiments of our group on the diaphragm of uremic rats showed that the relaxation time, which is related to the velocity of intracellular sequestration of calcium ions, is not prolonged in uremia (Senges, Ehe, Ritz: Uremic myopathy: Changes in contractile and membrane properties, in preparation). There are various explanations for the apparent discrepancy between the *in vitro* and *in vivo* findings. First of all, the first step of calcium ion sequestration in muscle is probably due to calcium ions binding to free calcium ionic binding sites of SR rather than to calcium ion transport [18]. Secondly, there is also evidence that in the absence of precipitating anions, i.e., under conditions more related to the *in vivo* situation, the calcium ion transport process of SR is different from that observed in the presence of these anions [19–21]. Since vesicles of uremic animals were able to lower ambient calcium ion concentration below 2×10^{-7}, the relaxation threshold of the actomyosin system, unimpaired relaxation is possible in spite of impaired calcium ion uptake. Total muscle calcium has not been measured in these experiments; however, a decrease of total muscle calcium might account for the ability of uremic muscle to efficiently lower cytosolic calcium ion concentration in spite of a diminished net calcium ion transport rate. Finally, it cannot be excluded that steps other than sarcoplasmic calcium ion transport (e.g., dissociation of calcium from troponin) are rate limiting in the relaxation process.

Biochemical consequences of uremia unrelated to the calcium ion transport defect. Experimental uremia is associated with increased blood urea concentrations, metabolic acidosis, secondary hyperparathyroidism, calorie and protein malnutrition, and uremic polyneuritis. These factors are unlikely to be the cause of the findings observed, since administration of urea in drinking water, induction of metabolic acidosis by ammonium chloride, and administration of bovine parathyroid hormone did not result in comparable changes of the vesicular function [22, 23]. The difference between control and uremic animals was still present when food was completely withheld; protein-calorie malnutrition can further be excluded, since pairfeeding was performed in all experiments reported above. Most likely, the vesicular transport defect is not the result of uremic polyneuritis, since the difference between uremic and control animals was still present when the hind limbs were paralyzed by sectioning of the sciatic nerve [22, 23].

Effect of vitamin D metabolites. In the above discussion, the system could be defined kinetically and a variety of artifacts or non-vitamin D related factors could be excluded as the cause of the transport defect in uremia. In the following we discuss the evidence that this defect is related to a disturbance of vitamin D metabolism. At the dose used, 1,25-$(OH)_2$-vitamin D_3 failed to increase significantly transport parameters in nonuremic control animals. This excludes the possibility that we are dealing only with a pharmacologic effect of 1,25-$(OH)_2$-vitamin D_3. Neither control nor uremic animals were vitamin D-depleted, since 25-(OH)-vitamin D levels were in the normal range. This excludes the possibility that we are dealing with repletion of vitamin D or 25-(OH)-vitamin D deficiency. Renal synthesis of 1,25-$(OH)_2$-vitamin D, the active metabolite of vitamin D, is impaired in renal insufficiency [24]. Deficiency of 1,25-$(OH)_2$-vitamin D appears to be the cause of the impairment of the function of the sarcoplasmic reticulum in uremia. Whereas in previous experiments, *in vivo* administration of vitamin D or 25-(OH)-vitamin D at the one dose level in the supraphysiological range prior to nephrectomy failed to correct the defect of calcium ion kinetics observed in uremia [25], repeated administration of 1,25-$(OH)_2$-vitamin D_3 at

the high dose level (as opposed to bolus injection of 1,25-$(OH)_2$-vitamin D_3 [25]) improved defective storing capacity, concentrating ability, and initial rate of calcium ion uptake. At the low dose level, only the storing capacity was consistently improved over the nontreated uremic animals. The higher dose of 1,25-$(OH)_2$-vitamin D_3 completely restored to normal all calcium ionic transport parameters. The greater sensitivity of storing capacity to 1,25-$(OH)_2$-vitamin D_3 may imply dependence of this parameter on membrane properties, binding or nucleation phenomena not reflected in the kinetics of the uptake experiment, or else it may result from the more favorable conditions under which storing capacity is measured (i.e., optimal ATP concentration and calcium ion gradient). The lower of the doses used was similar to the one which has recently been shown to improve the calcemic response of the skeleton to parathyroid hormone (PTH) infusion in uremic dogs [26].

As discussed above, the diminished rate of initial uptake of calcium ions results primarily from a diminished calcium ion influx rate constant (K_2). Therefore, the action of 1,25-$(OH)_2$-vitamin D_3 cannot be explained by an effect on passive calcium ionic permeability. The increase in passive efflux, though demonstrable in uremia [4], is unable to account for the diminished rate of initial calcium ion uptake for quantitative reasons. We are unable to decide whether the action of 1,25-$(OH)_2$-vitamin D_3 is due to the generation of additional transport sites or due to an increase in the rate constant, or due to both. A thermodynamic interpretation of the action of 1,25-$(OH)_2$-vitamin D_3 would be that the rate and efficiency of calcium ion transport is increased, i.e., the ability to convert chemical potential into osmotic potential. The effect of 1,25-$(OH)_2$-vitamin D_3 apparently is an indirect one, since *in vitro* incubation of the vesicles with 1,25-$(OH)_2$-vitamin D_3 in concentrations equivalent to circulating 1,25-$(OH)_2$-vitamin D_3 levels failed to correct the transport defect.

These results demonstrate that skeletal muscle is an important target organ for vitamin D. This is also suggested by the result of Birge and Haddad that 25-(OH)-vitamin D affects muscle metabolism [27] as well as by the findings of Curry, Francis and Smith that the function of the sarcoplasmic reticulum is impaired in vitamin D deficiency [28]. The sarcoplasmic reticulum, being a well established model of active transporting membranes, might well provide an effective *in vitro* model of a subcellular system to elucidate the multisystem effects of uremia on calcium ion transport.

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References

1. STANBURY, SW: Muscular disorders of metabolic bone disease. *Manch Med Gaz* 45: 16–23, 1965
2. BUNDSCHU HD, SUCHENWIRTH R, DURR F: Enzymhistologische Befunde an der Skelettmuskulatur des Menschen: II. Muskelschwund bei Niereninsuffizinez. *Klin Wochenschr* 48: 1389–1397, 1970
3. RITZ E, BUNDSCHU HD, HASSELBACH W: *Calcium Uptake by the Sarcoplasmic Reticulum in Experimental Uremia*, edited by CZITOBER H and ESCHBERGER J, Vienna, Facta Publication, 1973
4. HEIMBERG KW, MATTHEWS C, RITZ E, AUGUSTIN J, HASSELBACH W: Active Ca transport of the sarcoplasmic reticulum during experimental uremia: Changes in kinetics and lipid composition. *Eur J Biochem* 61: 207–213, 1976
5. EDELSTEIN S, CHARMAN M, LAWSON DE, KODICEK E: Competitive protein-binding assay for 25-hydroxycholecalciferol. *Clin Sci Mol Med* 46: 231–240, 1974
6. DE MEIS D, HASSELBACH W: Acetyl phosphate as substrate for Ca^{+2} uptake in skeletal muscle microsomes. *J Biol Chem* 246: 4759–4763, 1971
7. PULLMAN ME, PENEFSKY HS, DATTA A, RACKER E: Partial resolution of the enzymes catalyzing oxidative phosphorylation: I. Purification and properties of soluble, dinitrophenol-stimulated adenosine triphosphatase. *J Biol Chem* 235: 3322–3329, 1960
8. HASSELBACH W, MAKINOSE M: Uber den Mechanismus des Calciumtransports durch die Membranen des sarcoplasmatischen Reticulums. *Biochem Z* 339:94–111, 1963
9. AGOSTINI B, HASSELBACH W: Electron cytochemistry of calcium uptake in the fragmented sarcoplasmic reticulum. *Histochemie* 28:55–67, 1971
10. HASSELBACH W, MAKINOSE M: Die Calciumpumpe der "Erschlaffungsgrana" des Muskels und ihre Abhangigkeit von der ATP-Spaltung. *Biochem Z* 333:518–528, 1961
11. HASSELBACH W, MAKINOSE M: *The Reversal of the Sarcoplasmic Calcium Pump: Role of Membranes in Secretory Processes*, Amsterdam, North Holland, 1972, p. 158–169
12. *Handbook of Electroencephalography and Clinical Neurophysiology*. Edited by REMOND A, and STRUPPLER E, 1974, vol. 2, part D, p. 2–20
13. SRETER FA, GERGELY J: Comparative studies of the Mg activated ATPase activity and Ca uptake of fractions of white and red muscle homogenates. *Biochem Biophys Res Commun* 16: 438–443, 1964

14. HOERI W: Max-Planck-Institut fur Biophsik Lecture on Jan. 10, 1976, Frankfurt/Main, unpublished communication
15. DE BOLAND AR, JILKA RL, MARTONOSE AN: Passive Ca^{+2} permeability of phospholipid vesicles and sarcoplasmic reticulum membranes. *J Biol Chem* 250:7501–7510, 1975
16. AGOSTINI B, SRETER FA, GERGELY J: Electron cytochemistry of Ca-uptake and ATPase activity of fragmented sarcoplasmic reticulum after denervation, in *IVth European Regional Conference on Electron Microscopy*, edited by BOCCIARELLI DS, Roma, Tipografia Poliglotta Vaticano, 1968, p. 285–286
17. SERAYDARIAN K, MOMMAERTS WFHM, WALLNER A, GUILLORY RF: An estimation of the true inorganic phosphate content of frog sartorius muscle. *J Biol Chem* 236:2071–2075, 1961
18. HASSELBACH W: Release and uptake of calcium by the sarcoplasmic reticulum, in *Kolloquium der Gesellschaft fur Physiologische Chemie*, edited by HEILMEYER L, Heidelberg, Springer Verlag, p. 141–161, in press
19. WEBER A, HERZ R, REISS I: Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. *Biochem Z* 345:329–369, 1966
20. MERMIER P, HASSELBACH W: The biphasic Ca^{2+}-uptake by the fragmented sarcoplasmic reticulum. *Z Naturforsch* 30 (c):593–599, 1975
21. MERMIER P, HASSELBACH W: The effect of calcium and phosphate on the biphasic calcium uptake by the sarcoplasmic reticulum. *Z Naturforsch* 30 (c):777–780, 1975
22. MATTHEWS C, RITZ E, HASSELBACH W, BUNDSCHU HD: Veranderung an der quergestreiften Muskulatur be experimenteller Uramie. *Verh Dtsch Ges Inn Med* 79:705–708, 1973
23. RITZ E, BUNDSCHU HD, MATTHEWS C, SENGES J, HEIMBERG KW, EHE L, AUGUSTIN J: Veranderungen an der quergestreiften Muskulatur bei chronischer Niereninsuffizienz, in *Vth Symposium, Aktuelle Probleme der Dialyseverfahren und der Niereninsuffizienz, Innsbruck*, edited by DIETRICH PV, SKRABAL F and STULENGER WE, Friedberg (Hessen), Verlag Bindernagel, 1974, p. 472–483
24. FRASER DR, KODICEK E: Unique biosynthesis by kidney of biologically active vitamin D metabolite. *Nature* 228:764–766, 1970
25. RITZ E: Vitamin D and Problems Related to Uremic Bone Diseases, in *2nd Proceedings, Workshop on Vitamin D*, Wiesbaden, West Germany, Oct. 1974, edited by AW NORMAN et al, 1975
26. MASSRY SG, STEIN R, GARTY J, ARIEFF AI, COBURN JW, NORMAN AW, FRIEDLER RM: Skeletal resistance to the calcemic action of parathyroid hormone in uremia: Role of $1,25(OH)_2D_3$. *Kidney Int* 9:467–474, 1976
27. BIRGE SJ, HADDAD JG: 25-Hydroxycholecalciferol stimulation of muscle metabolism. *J Clin Invest* 56:1100–1107, 1975
28. CURRY O, FRANCIS MJO, SMITH R: The effects of vitamin D deficiency on the isolated sarcoplasmic reticulum of muscle.*Clin Sci Mol Med* 46:7P 1974
29. SCHWARZENBACH G: *Die Komplexometrische Titration.* Stuttgart, F. Enke, 1960